Larvicidal potential of commercially available pine (*Pinus longifolia*) and cinnamon (*Cinnamomum zeylanicum*) oils against dengue fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae)

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Abstract: The aromatic nature of pine and cinnamon oils has established them as good adult repellents but their larvicidal efficacy against mosquitoes has not been explored much. Keeping this in view, laboratory studies were conducted to uncover the larvicidal potential of commercially available pine (*Pinus longifolia*) oil and cinnamon (*Cinnamomum zeylanicum*) oil against the early 4th instar larvae of *Aedes aegypti*, originating from Delhi, India. The larvicidal effects were investigated in terms of the larval mortality, behavioural and morphological changes, if any. Our studies revealed the larvicidal efficiency of both the oils against 4th instar larvae of *Ae. aegypti*, the pine oil proving to be more effective than the cinnamon oil. The LC_{50} and LC_{70} values exhibited by pine oil were 0.33093 mg/L and 0.54476 mg/L, respectively, while the values obtained with cinnamon oil were 0.63159 mg/L and 0.77736 mg/L, respectively. It was further observed that at LC_{90} the larvicidal potential of cinnamon oil surpassed that of the pine oil, exhibiting a value of 1.11879 mg/L as in comparison to 1.04915 mg/L shown by pine oil. The behavioural changes as excitation, restlessness, tremors, and convulsions followed by paralysis observed in the treated larvae suggest a probable effect of the oils on their neuromuscular system. Microscopic study of morphological alterations in the treated larvae revealed that most of their organs had a normal structural appearance as that of controls except the little internal shrinkage in anal gills leading to the structural deformity. This indicates the anal gills as the probable action sites of the oil extracts and dysfunction of the gills leading to larval mortality. The potential of oils as new types of larvicides for the control of mosquitoes are explored.

Key words: *Aedes aegypti*; pine; cinnamon; larvicidal potential; anal gills; convulsions

1 INTRODUCTION

The vector-borne diseases caused by mosquitoes are one of the major health problems in many countries (Kannathasan *et al.*, 2007). Besides being a summer nuisance, mosquitoes pose some major public health problems, carrying pathogens of the deadly diseases such as dengue, malaria, encephalitis, yellow fever, chikungunya, and filariasis. These vectors occur mainly in tropical countries where more than two billion people live in endemic regions with about one million deaths been claimed yearly from malaria and filariasis (Southgate, 1984; WHO, 2005). In the absence of effective preventive measures or vaccine, the best approach should be the interruption of disease transmission by either killing, preventing mosquitoes from biting people or by killing the larva at the breeding sites of vectors. The control of mosquitoes has been complicated because the repeated use of chemical insecticides has disrupted natural biological control systems sometimes resulting in the widespread development of resistance. These problems have warranted the need for search and development of alternative strategies using eco-friendly products which are environmentally safe, biodegradable and low cost larvicides and adultcides for killing larval and adult mosquitoes respectively from natural sources (Mittal and Subbarao, 2003). Natural products are the best option because of their less harmful nature to environment and non-targeted organisms. Plants offer an alternative source of insect-control agents because they contain a range of bioactive chemicals, many of which are selective and have little or no harmful effect on non-target organisms and the environment (Arnason *et al.*, 1989; Hedlin *et al.*, 1997). In recent years, much effort has, therefore, been focused on plant extracts or phytochemicals as potential sources of mosquito control agents or as lead compounds (Sukumar *et al.*,

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1991; Ansari *et al.*, 2005; Singh *et al.*, 2006). Several extracts and compounds from different plant families have been evaluated to show new and promising larvicides (Markouk *et al.*, 2000; Prabakar and Jebanesan, 2004; Mohan and Ramaswamy, 2007; Innocent *et al.*, 2008a, 2008b), however very few plant products have been developed for controlling mosquitoes. The plant oils have received much attention as potentially useful bioactive compounds against insects showing a broad spectrum of activity against insects, low mammalian toxicity and degrading rapidly in the environment (Kim *et al.*, 2001). Keeping in mind the availability, low budget and less environmental impact, the experiments were performed on the commonly available essential oils already in domestic use to assess their mosquiticidal potential.

Pine oil and cinnamon oil show promise as a great-smelling, environmentally friendly pesticide, with the ability to kill mosquito larvae (Cheng *et al.*, 2004; Ansari *et al.*, 2005). *Pinus longifolia* (Family: Pinaceae) commonly known as pine, yields oil which is traditionally used for the protection from mosquito bites. It is also used as a herbal medicine in some rural areas in India. In addition to oil, resins of the pine have been used as a mounting medium for the preservation of insects (Ansari *et al.*, 2005). Cinnamon oil is obtained from the bark and leaves of cinnamon tree, *Cinnamomum zeylanicum*. Cinnamon, due to its medicinal properties, has been used in many cultures for treating a variety of health disorders. Cinnamaldehyde is the main constituent in cinnamon leaf oil and is used worldwide as a food additive and flavouring agent, and as mosquito repellent (Cheng *et al.*, 2004). Much work has not been carried out regarding the larvicidal effects of the pine and cinnamon oil against mosquitoes. Present studies, thus, were carried out to study the larvicidal potential of the commercially available pine and cinnamon oils against dengue fever mosquito, *Ae. aegypti*.

2 MATERIALS AND METHODS

2.1 Mosquito culture

The present investigations employ the third instar and the early fourth instar larvae of *Ae. aegypti* originated from field-collected engorged female adults from Delhi, India. The colony was maintained in an insectary without any insecticide exposure at 28 ± 1℃, 80% ±5% RH and 14L∶10D photoperiod (Kumar *et al.*, 2002). Adults were supplied with soaked deseeded raisins. Periodic blood meals were provided to female mosquitoes for egg maturation by keeping restrained albino rats in the cages. The eggs were collected in an enamel bowl lined with Whatman filter paper and were allowed to hatch in enamel trays filled with de-chlorinated water. Larvae were fed upon a mixture of yeast powder and grinded dog biscuits. The pupae formed were collected in enamel bowls and transferred to the cloth cages for adult emergence.

2.2 Larvicidal bioassay

The larvicidal bioassay was performed at 28 ± 1℃ on the *Ae. aegypti* larvae in accordance with the WHO method for mosquito larvae (Kumar *et al.*, 2002). The graded series of each oil was prepared using ethanol as the solvent. The early 4th instar larvae of *Ae. aegypti*, in batches of 25, were taken in plastic bowls containing 99 mL of distilled water and transferred to glass jar containing 150 mL of distilled water and 1 mL of the particular concentration of oil. Four replicates were carried out simultaneously for each dilution making a total of 100 larvae for each concentration. Controls were exposed to the solvent, *i.e.* ethanol alone.

2.3 Behavioural study

Immediately after treatment, symptoms in treated larvae were observed and recorded at frequent time intervals. During the treatment period no food was offered to the larvae. The larvae were considered moribund if, at the end of 24 h, they showed no sign of swimming movements even after gentle touching with a glass rod. The dead and moribund larvae were recorded after 24 h as larval mortality.

2.4 Morphological study

The dead larvae treated with a lethal dosage (LC_{99}) of each oil, separately, were mounted with Hoyer's medium on a microscope slide and scrutinized for morphological alterations under light microscopy. Morphological changes in body segments including the head, thorax, and abdomen, and other organs such as the eyes, antennae, mouth brushes, setae, saddle, and anal gills were observed, photographed, and compared with those of the controls.

2.5 Data statistics and analysis

The larvicidal tests with more than 20% mortality in controls and pupae formed were discarded and repeated again. If the control mortality ranged between 5% and 20%, it was corrected using Abbott's formula (Abbott, 1925).

$$\text{Corrected mortality} = \frac{(\%\ \text{Test mortality} - \%\ \text{Control mortality}) \times 100}{100 - \%\ \text{Control mortality}}$$

The data were subjected to regression analysis using computerized SPSS 11.5 Programme. The

LC_{30}, LC_{50}, LC_{70}, LC_{90} and LC_{99} values with 95% fiducial limits were calculated in each bioassay to measure difference between the test samples. The results obtained with different oils were analyzed using Student's *t*-test with statistical significance considered for $P \leqslant 0.05$.

3 RESULTS

3.1 Larvicidal bioassay

The larvicidal activities of the pine and cinnamon oil, commercially available in New Delhi, India, on early 4th instar larvae of *Ae. aegypti* are presented in Table 1 and 2. Bioassays performed with the two kinds of oils revealed their potency to kill mosquito larvae at quite low concentrations. The treatment resulted in complete mortality with no pupal or adult emergence. The control or untreated group did not exhibit any mortality within 24 h and the larvae developed into pupae and then adults within 48 – 72 h.

Our investigations clearly showed that at LC_{30} to LC_{50} levels, the pine oil was 1.8 to 2.5-fold more effective against the larvae of *Ae. aegypti* than the cinnamon oil exhibiting an LC_{30} value of 0.201 mg/L and an LC_{50} value of 0.330 mg/L (Table 1). These values, however, obtained with cinnamon oil were 0.513 mg/L and 0.613 mg/L, respectively (Table 2). Likewise, at LC_{70} pine oil proved to be almost 1.5 times more potent than cinnamon oil. However, statistical analysis clearly showed that the LC values obtained with pine oil treatment were significantly different except at LC_{99} whereas the values obtained after larvicidal bioassay with cinnamon oil were not significantly different suggesting pine oil to be more efficient than cinnamon oil. Nevertheless, the results clearly show a gradually increasing effectiveness of cinnamon oil than the pine oil with the increase in lethal concentration (Fig. 1). It is clear from the Fig. 1 that the potency of cinnamon oil surpassed that of pine oil at a concentration level somewhere between LC_{85} and LC_{90}. It was found to be 1.06-fold more effective than pine oil at LC_{90} while it became 1.9-fold more potent at LC_{99} level.

Table 1 Larvicidal effects of pine oil on the early 4th instar larvae of *Aedes aegypti*

Concentration values	LC_{30} (mg/L)	LC_{50} (mg/L)	LC_{70} (mg/L)	LC_{90} (mg/L)	LC_{99} (mg/L)	χ^2 (*df*)	Regression equation	*S. E.*
Lethal concentration	0.20103	0.33093	0.54476	1.11879	3.02017			
Lower fiducial limit	0.12767	0.25486	0.43907	0.80115	1.70648	3.676(3)	$Y = 1.163 + 2.422X$	0.210
Upper fiducial limit	0.25997	0.40915	0.74616	2.13553	9.81049			

Table 2 Larvicidal effects of cinnamon oil on the early 4th instar larvae of *Aedes aegypti*

Concentration values	LC_{30} (mg/L)	LC_{50} (mg/L)	LC_{70} (mg/L)	LC_{90} (mg/L)	LC_{99} (mg/L)	χ^2 (*df*)	Regression equation	*S. E.*
Lethal concentration	0.51315	0.63159	0.77736	1.04915	1.58680			
Lower fiducial limit	0.44934	0.55608	0.67215	0.86589	1.20983	0.426(3)	$Y = 1.160 + 5.814X$	0.289
Upper fiducial limit	0.58448	0.74222	0.96501	1.43880	2.53451			

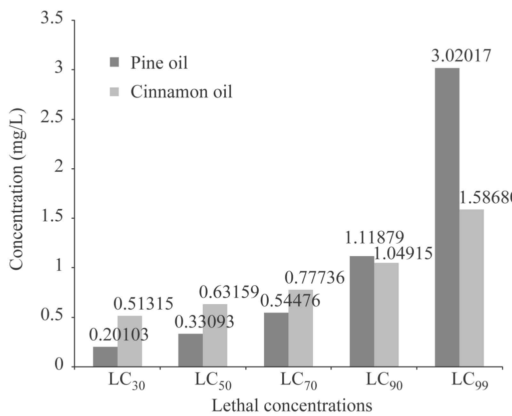

Fig. 1 Comparison of lethal concentrations of pine oil and cinnamon oil against the early 4th instar larvae of *Aedes aegypti*

3.2 Behavioural study

The symptomatological observations on the larvae treated with the oils at LC_{99} revealed a particular pattern in their behaviour (Table 3). The noticeable observation was the earlier appearance of symptoms in cinnamon-treated larvae than the pine-treated larvae. After immediate exposure to both the oils, all larvae were active with clearly visible movements. Initial abnormal excitation, restlessness, and sluggishness were observed after 2 – 4 min of exposure which continued and persisted till 10 – 35 min after which other abnormal motions such as a coiling movement were seen. In both cases, the treated larvae frequently sank down and floated up again quickly. Thereafter more toxic symptoms including tremor and convulsion appeared followed by paralysis. More than one-half of the larvae were paralyzed and had sunk to the bottom of the bowl after 2 – 3 h. Moribund or dead larvae were

Table 3 Symptomatological observations on the early 4th instar larvae of *Aedes aegypti* after treatment with cinnamon oil and pine oil at LC_{99} level

S. no.	Symptoms observed in the larvae treated at LC_{99} of the oil	Time of initiation of symptoms	
		Cinnamon oil (LC_{99} = 3.02017 mg/L)	Pine oil (LC_{99} = 1.58680 mg/L)
1	Initial abnormal excitation, restlessness, and sluggishness	2 min of exposure	4 min of exposure
2	Abnormal motions such as a coiling movement, quick wriggling action	10 – 20 min of exposure	30 – 35 min of exposure
3	Toxic symptoms with little tremor and convulsions	20 – 25 min of exposure	35 – 40 min of exposure
4	Excitation, restlessness, tremors and convulsions followed by paralysis	40 min of exposure	60 min of exposure
5	50% of the larvae paralyzed and sank to the bottom of the bowl	2 h of exposure	3 h of exposure
6	100% mortality	24 h of exposure	24 h of exposure

increasingly found each hour leading to 100% mortality after 24 h.

3.3 Morphological study

The morphological alterations of treated 4th instar larvae, when observed revealed that all of the organs, except anal gills, had a normal structural appearance. Under light both treated and control larvae showed similarities in morphological architecture and cuticular sculpturing of the body segments, and other organs such as the eyes, antennae, mouthbrushes, setae, saddle, siphon, and ventral brushes. However, the anal gills of all treated larvae showed a structural alteration in the internal structures with distinct shrinkage, while the external features were normal in appearance (Fig. 2).

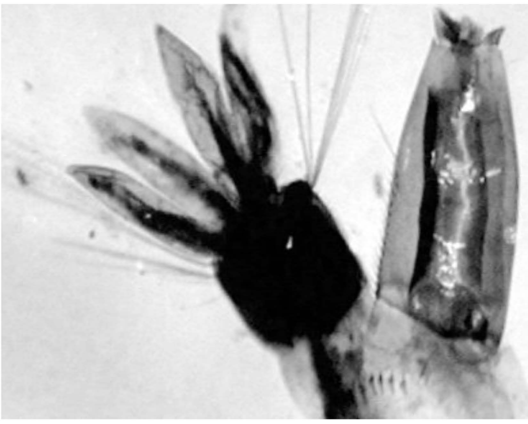

Fig. 2 Light micrograph of anal gills of oil-treated *Aedes aegypti* larvae showing normal external appearance and shrinkage in internal structure

4 DISCUSSION

4.1 Larvicidal bioassay

Essential oils extracted from different plants have been reported to have larvicidal and repellent properties against *Ae. aegypti* (Cavalcanti *et al.*, 2004; Amer and Mehlhorn 2006; Morais *et al.*, 2006; Silva *et al.*, 2008; Waliwitiya *et al.*, 2009). However, very few reports are available about the potential of pine and cinnamon oil as mosquito larvicide agent. The present investigations were performed to assess the potential of commercially available pine oil and cinnamon oil as larvicidal agent against *Ae. aegypti*.

Our studies revealed the pine oil as the efficient larvicidal agent against *Ae. aegypti*. On exposure to commercially available pine oil, the early 4th instar larvae showed an LC_{50} value of 0.330 mg/L which significantly increased to 1.118 mg/L at LC_{90}. Similar results were reported by Ansari *et al.* (2005) who also found the commercially available pine oil highly effective against an Indian strain of *Ae. aegypti* though the LC_{50} (82.1 mg/L) and LC_{90} value (252 mg/L) accounted by them were much higher than obtained by us. They established the larvicidal potential of pine oil against *Cx. quinquefasciatus* (LC_{50}: 85.7 mg/L; LC_{90}: 283.4 mg/L) and *An. stephensi* (LC_{50}: 112.6 mg/L; LC_{90}: 329.5 mg/L) too, though the efficacy was found to be less than that against *Ae. aegypti*. They further suggested that despite of larvicidal potential of pine oil, it would not be practical to use it as a larvicide in non-potable water in large breeding habitats as it requires very high doses to be effective, however, it might be used as larvicide selectively in small breeding places such as in domestic and peri-domestic containers, desert coolers, *etc.*, where water is stagnant. On the other hand, our studies have revealed quite low doses of pine oil to be effective against the dengue fever mosquito larvae and thus it can be used in large breeding habitats. Still it is advisable that before reaching any conclusion field experiments need to be conducted.

Similar results were found when the early fourth instar larvae of *Ae. aegypti* were tested with cinnamon oil. An interesting observation was that at lower LC values, pine oil was found more effective

(2.5-fold at LC_{30}, 1.8-fold at LC_{50}) than cinnamon oil, while gradually more efficacy was exhibited by cinnamon oil (1.06-fold at LC_{90}). Further research is needed to find out the reasons for this finding. Cheng *et al.* (2004) compared the leaf essential oils from eight provenances of indigenous cinnamon (*Cinnamomum osmophloeum* Kaneh.) and reported that the essential oils of cinnamaldehyde type and cinnamaldehyde/cinnamyl acetate type had an excellent inhibitory effect against the 4th instar larvae of *Ae. aegypti* with LC_{50} values of 36 mg/L and 44 mg/L; and LC_{90} values of 79 mg/L and 85 mg/L, respectively. Cinnamaldehyde was reported to possess the best mosquito larvicidal activity against *Ae. aegypti*, with an LC_{50} of 29 mg/L and LC_{90} of 48 mg/L (Cheng *et al.*, 2004). The larvicidal activity of cinnamon and other oils were recorded by Zhu *et al.* (2006, 2008) against 4th instars of *Ae. albopictus*, *Ae. aegypti*, and *Culex pipiens pallens*.

4.2 Behavioural study

The gradual abnormal behaviour in the motion of treated larvae followed by excitation, convulsions, paralysis, and then resulting in 100% kill indicates a delayed type of larval killing from the plant oils. In fact, pine oil proved to have a more delayed effect in comparison to the cinnamon oil. These observations correspond to the earlier studies carried out to evaluate the larvicidal potential of plant-derived materials against some mosquito species (Choochote *et al.*, 2004, 2005; Dharmagadda *et al.*, 2005; Chaithong *et al.*, 2006). These studies conducted with plant oils or extracts of *Kaempferia galanga*, *Tagetes patula*, *Apium graveolens*, *Curcuma aromatica*, and *Piper* species showed similar symptoms in the exposed larvae though the time for the appearance of the toxic symptoms was relatively different. The observed symptoms being similar to those of nerve poisons, though slow, suggest the probable toxic effect of the plants on the neuromuscular system.

4.3 Morphological study

Our studies also showed the shrinkage in the internal membrane of the anal gills of the *Ae. aegypti* larvae treated with both the oils. These investigations are similar to that of Chaithong *et al.* (2006) who reported the remarkable shrinkage in the internal structure of anal papillae in the larvae of a Thailand strain of *Ae. aegypti* when treated with ethanolic extracts of black pepper, while most of the other organs of dead larvae had a normal appearance. Likewise, *Cx. quinquefasciatus* larvae treated with ethanolic extract of *Kaempferia galanga* revealed the severely morphological disruption of anal papillae with a shrunken cuticle border and destroyed surface with loss of ridge-like reticulum under light and scanning electron microscopy, respectively (Insun *et al.*, 1999). Green *et al.* (1991) also reported swollen anal papillae in *Ae. aegypti* larvae after treatment with whole oil of *Tagetes minuta*. It was suggested that structural deformation of anal papillae probably led to their dysfunction, which may be intrinsically associated with the death of mosquito larvae (Chaithong *et al.*, 2006). Earlier, it was reported that uptake and elimination of most ions in mosquito larvae occur via the anal papillae, which was markedly reduced or lost in papilla-less larvae (Garrett and Bradley, 1984; Clements, 1992). This indicated that the lack or dysfunction of the anal papillae probably led to an interruption of the osmotic and ionic regulation. However, the microscopic study alone cannot ascertain the cause of the larvicidal effects of the oils, which may be at physiological or biochemical level. Extensive and further research is required to investigate the site of action of the oils.

4.4 Conclusion

Our studies prove the potential of pine oil and cinnamon oil as the larvicidal agent against *Ae. aegypti*. The pine oil proved to be apparently more effective at lower LC values while gradually cinnamon oil proved to be more potent than pine oil. The toxic symptoms and deformity in anal gills suggest different modes of action of oils. Further research is needed to explore the action site of oils. The studies suggest that a formulation using the bioactive constituent of investigated oils could be used against mosquito larvae without the potential for adverse health effects and an added bonus of a pleasant smell. Further investigations are required to identify the bioactive constituents present in these oils possessing mosquicidal properties.

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市售长叶松油和锡兰肉桂油对登革热媒蚊埃及伊蚊的灭幼效果

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摘要: 松油和桂皮油由于具有芳香性气味, 因而成为良好的成虫驱避剂, 但是关于它们对蚊虫的杀幼虫作用研究不多。为揭示市售的长叶松 *Pinus longifolia* 油和锡兰肉桂 *Cinnamomum zeylanicum* 油对来源于印度德里的埃及伊蚊 *Aedes aegypti* 4 龄幼虫的毒杀潜力, 我们进行了室内研究, 以幼虫死亡率及行为改变和形态改变等指标评估其杀幼虫潜力。结果表明: 两种油对埃及伊蚊 4 龄幼虫均具有毒杀作用, 且松油的灭幼效果优于桂皮油。松油的 LC_{50} 和 LC_{70} 值分别为 0.33093 mg/L 和 0.54476 mg/L, 而桂皮油的 LC_{50} 和 LC_{70} 值分别为 0.63159 mg/L 和 0.77736。进一步观察发现, LC_{90} 剂量下桂皮油的杀幼虫潜力强于松油, 其 LC_{90} 为 1.11879 mg/L, 而松油的 LC_{90} 为 1.04915 mg/L。在处理的幼虫中观察到行为改变, 如兴奋、坐立不安、颤抖、痉挛然后瘫痪, 说明这两种油可能对其神经肌肉系统产生了影响。显微观察处理幼虫的形态改变发现, 与对照相比, 大多数器官的外观正常, 只是肛鳃略为内收缩而引起结构畸形, 提示肛腮可能是这两种油的作用位点, 腮的功能异常引起了幼虫死亡。这两种油品可开发用作防治蚊虫的新型杀幼虫药剂。

关键词: 埃及伊蚊; 松树; 肉桂; 杀幼虫潜力; 肛腮; 痉挛

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